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<p>(54) Title: WOUND HEALING</p> <p>(57) Abstract</p> <p>Healing an external wound or regenerating bone of a mammal by administering to the mammal a composition containing purified platelet/derived growth factor and purified transforming growth factor alpha.</p>		

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WOUND HEALING

Background of the Invention

This invention relates to healing wounds.

Growth factors are polypeptide hormones which
5 stimulate a defined population of target cells.
Examples of growth factors include platelet-derived
growth factor (PDGF), insulin-like growth factor
(IGF-I), transforming growth factor beta (TGF- β),
transforming growth factor alpha (TGF- α), epidermal
10 growth factor (EGF), and fibroblast growth factor
(FGF). PDGF is a cationic, heat-stable protein found in
the granules of circulating platelets which is known to
stimulate in vitro protein synthesis and collagen
production by fibroblasts. It is also known to act as
15 an in vitro mitogen and chemotactic agent for
fibroblasts, and smooth muscle cells.

It has been proposed to use PDGF to promote in vivo wound healing. For example, Grotendorst (1984) J. Trauma 24:549-52 describes adding PDGF to Hunt-Schilling
20 wire mesh chambers impregnated with a collagen gel and
implanted in the backs of rats; PDGF was found to
increase the amount of new collagen synthesized.
However, Leitzel et al. (1985) J. Dermatol. Surg. Oncol.
11:617-22 were unable to accelerate normal wound healing
25 in hamsters using PDGF alone or in combination with FGF
and EGF.

Michaeli, et al. (1984) In Soft and Hard Tissue Repair (Hunt, T.K. et al., Eds), Praeger Publishers, New
York, pp. 380-394, report that application of a
30 partially purified preparation of PDGF obtained from
platelet-rich plasma stimulated angiogenesis when

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implanted in rabbit corneas. Because PDGF is not an angiogenic growth factor the investigators suggested that an unknown factor in their partially purified PDGF preparation was responsible for the angiogenic effect.

- 5 Schultz, G.S. et al. (1987) Science reported that local application of TGF- α to partial thickness skin burns in pigs accelerated epidermal regeneration, in comparison with untreated burns.

Summary of the Invention

- 10 In general, the invention features healing an external wound in a mammal, e.g., a human patient, by applying to the wound an effective amount of a composition that includes a combination of purified PDGF and purified TGF- α . Preferably, the TGF- α is human
- 15 TGF- α but can also be of another mammalian species, e.g., rat. The TGF- α can be isolated from natural sources or, more preferably, produced by recombinant cells or solid phase peptide synthesis. The composition of the invention aids in healing the wound, at least in
- 20 part, by promoting the growth of epithelial and connective tissue and the synthesis of total protein and collagen. Wound healing using the composition of the invention is more effective than that achieved in the absence of treatment (i.e., without applying exogenous
- 25 agents) or by treatment with purified PDGF alone, or purified TGF- α alone.

- In preferred embodiment of the invention, the composition is prepared by combining, in a pharmaceutically acceptable carrier substance, e.g.,
- 30 commercially available inert gels or liquids (e.g., saline supplemented with albumin or methyl cellulose), purified PDGF and TGF- α (both of which are commercially available). Most preferably purified PDGF and TGF- α are combined in a weight-to-weight ratio of between 1:4

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and 25:1, preferably between 1:2 and 10:1, and more preferably 1:1 or 2:1. The purified PDGF may be obtained from human platelets or by recombinant DNA technology. Thus, by the term "PDGF" we mean both platelet-derived and recombinant materials of mammalian, preferably primate, origin; most preferably, the primate is a human, but can also be a chimpanzee or other primate. Recombinant PDGF can be recombinant heterodimer, made by inserting into cultured prokaryotic or eukaryotic cells DNA sequences encoding both subunits, and then allowing the translated subunits to be processed by the cells to form heterodimer, or DNA encoding just one of the subunits (preferably the beta or "2" chain) can be inserted into cells, which then are cultured to produce homodimeric PDGF (PDGF-1 or PDGF-2 homodimer).

The term "purified" as used herein refers to PDGF or TGF- α which, prior to mixing with the other, is 95% or greater, by weight, PDGF or TGF- α , i.e., is substantially free of other proteins, lipids, and carbohydrates with which it is naturally associated.

A purified protein preparation will generally yield a single major band on a polyacrylamide gel for each PDGF or TGF- α component. Most preferably, the purified PDGF or TGF- α used in the composition of the invention is pure as judged by amino-terminal amino acid sequence analysis.

The composition of the invention provides a fast, effective method for healing external wounds of mammals, e.g., bed sores, lacerations and burns. The composition enhances connective tissue formation compared to natural healing (i.e. no exogenous agents added) or pure PDGF or TGF- α alone. Unlike pure PDGF alone, the composition promotes a significant increase

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in both new connective tissue and epithelial tissue.
The epithelial layer obtained is thicker than that
created by natural healing or by TFG- α alone, and also
contains more epithelial projections connecting it to
5 the new connective tissue; it is thus more firmly bound
and protective.

Other features and advantages of the invention
will be apparent from the following description of the
preferred embodiments thereof, and from the claims.

10 Description of the Preferred Embodiments

We now describe preferred embodiments of the
invention.

External wounds, e.g., bed sores and burns, are
treated, according to the invention, with PDGF/TGF- α
15 mixtures prepared by combining pure PDGF and TGF- α .
Chemically synthesized human and rat TGF- α are
commercially available from Peninsula Laboratories
(Belmont, CA). Purified recombinant PDGF and purified
PDGF derived from human platelets are commercially
20 available from PDGF, Inc. (Boston, MA), Collaborative
Research (Waltham, MA), and Amgen Corp. (Thousand Oaks,
CA). Purified PDGF can also be prepared as follows.

Five hundred to 1000 units of washed human
platelet pellets are suspended in 1M NaCl (2ml per
25 platelet unit) and heated at 100°C for 15 minutes. The
supernatant is then separated by centrifugation and the
precipitate extracted twice with the 1M NaCl.

The extracts are combined and dialyzed against
0.08M NaCl-0.01M sodium phosphate buffer (pH 7.4) and
30 mixed overnight at 4°C with CM-Sephadex C-50
equilibrated with the buffer. The mixture is then
poured into a column (5 x 100 cm), washed extensively
with 0.08M NaCl-0.01M sodium phosphate buffer (pH 7.4),
and eluted with 1M NaCl while 10 ml fractions are
35 collected.

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Active fractions are pooled and dialyzed against 0.3M NaCl-0.01M sodium phosphate buffer (pH 7.4), centrifuged, and passed at 4°C through a 2.5 x 25 cm column of Blue Sepharose (Pharmacia) equilibrated with 0.3M NaCl-0.01M sodium phosphate buffer (pH 7.4). The column is then washed with the buffer and partially purified PDGF eluted with a 1:1 solution of 1M NaCl and ethylene glycol.

The partially purified PDGF fractions are diluted (1:1) with 1M NaCl, dialyzed against 1M acetic acid, and lyophilized. The lyophilized samples are dissolved in 0.8M NaCl-0.01M sodium phosphate buffer (pH 7.4) and passed through a 1.2 x 40 cm column of CM-Sephadex C-50 equilibrated with the buffer. PDGF is then eluted with a NaCl gradient (0.08 to 1M).

The active fractions are combined, dialyzed against 1M acetic acid, lyophilized, and dissolved in a small volume of 1M acetic acid. 0.5 ml portions are applied to a 1.2 x 100 cm column of Biogel P-150 (100 to 200 mesh) equilibrated with 1M acetic acid. The PDGF is then eluted with 1M acetic acid while 2 ml fractions are collected.

Each active fraction containing 100 to 200 mg of protein is lyophilized, dissolved in 100 ml of 0.4% trifluoroacetic acid, and subjected to reverse phase high performance liquid chromatography on a phenyl Bondapak column (Waters). Elution with a linear acetonitrile gradient (0 to 60%) yields pure PDGF.

PDGF made by recombinant DNA technology can be prepared as follows:

Platelet-derived growth factor (PDGF) derived from human platelets contains two polypeptide sequences (PDGF-1 and PDGF-2 polypeptides; Antoniades, H.N. and Hunkapiller, M. (1983) Sci nc 220:963-965). PDGF-1 is

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encoded by a gene localized in chromosome 7 (Betsholtz, C. et al., Nature 320:695-699), and PDGF-2 is encoded by the sis oncogene (Doolittle, R. et al. (1983) Science 221:275-277) localized in chromosome 22 (Dalla-Favera, R. (1982) Science 218:686-688). The sis gene encodes the transforming protein of the Simian Sarcoma Virus (SSV) which is closely related to PDGF-2 polypeptide. The human cellular c-sis also encodes the PDGF-2 chain (Rao, C.D. et al. (1986) Proc. Natl. Acad. Sci. USA 83:2392-2396). Because the two polypeptide chains of PDGF are coded by two different genes localized in separate chromosomes, the possibility exists that human PDGF consists of a disulfide-linked heterodimer of PDGF-1 and PDGF-2, or a mixture of the two homodimers (homodimer of PDGF-1 and homodimer of PDGF-2), or a mixture of the heterodimer and the two homodimers.

Mammalian cells in culture infected with the Simian Sarcoma Virus, which contains the gene encoding the PDGF-2 chain, were shown to synthesize the PDGF-2 polypeptide and to process it into a disulfide-linked homodimer (Robbins, K. et al. (1983) Nature 305:605-608). In addition, PDGF-2 homodimer reacts with antisera raised against human PDGF. Furthermore, the functional properties of the secreted PDGF-2 homodimer are similar to those of platelet-derived PDGF in that it stimulates DNA synthesis in cultured fibroblasts, it induces phosphorylation at the tyrosine residue of a 185 kd cell membrane protein, and it is capable of competing with human (^{125}I)-PDGF for binding to specific cell surface PDGF receptors (Owen, A. et al. (1984) Science 225:54-56). Similar properties were shown for the sis/PDGF-2 gene product derived from cultured normal human cells (for example, human arterial endothelial cells), or from human malignant cells expressing the

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sis/PDGF-2 gene (Antoniades, H. et al. (1985) Cancer Cells 3:145-151).

- The recombinant PDGF-2 homodimer (referred to as recombinant PDGF herein) is obtained by the introduction of cDNA clones of c-sis/PDGF-2 gene into mouse cells using an expression vector. The c-sis/PDGF-2 clone used for the expression was obtained from normal human cultured endothelial cells (Collins, T., et al. (1985) Nature 216:748-750).

10 Wound Healing

To determine the effectiveness of PDGF/TGF- α mixtures in promoting wound healing, the following experiments were performed.

- Young white Yorkshire pigs (Parson's Farm, Hadley, MA) weighing between 10 and 15 kg were fasted for at least 6 hours prior to surgery and then anesthetized. Under aseptic conditions, the back and thoracic areas were clipped, shaved, and washed with mild soap and water. The area to be wounded was then disinfected with 70% alcohol.

- Wounds measuring 1 cm x 2 cm were induced at a depth of 0.5 mm using a modified Castroviejo electrokeratome (Storz, St. Louis, MO, as modified by Brownells, Inc.). The wounds resulted in complete removal of the epithelium, as well as a portion of the underlying dermis (comparable to a second degree burn injury). Individual wounds were separated by at least 15 mm of unwounded skin. Wounds receiving identical treatment were organized as a group and separated from other groups by at least 3 cm. Wounds receiving no growth factor treatment were separated from wounds receiving such treatment by at least 10 cm.

The wounds were treated directly with a single application of the following growth factors suspended in

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biocompatible gel: 1) 500 ng pure human PDGF (purified by high performance liquid chromatography) or recombinant PDGF alone; 2) 500 ng pure recombinant PDGF in combination with each of the following: a) 500 ng human TGF- α ; b) 500 ng rat TGF- α ; 3) 500 ng human or rat TGF- α alone.

Following wounding, biopsy specimens were taken on days 3 through 10. Biopsy specimens for histologic evaluation were taken as wedges approximately 3 mm deep and placed in 10% formalin. Specimens for biochemical analysis and autoradiography were obtained using an electrokeratome. The final dimensions of the specimens were 1.5 mm x 10 mm x 1.5 mm. Three specimens per wound were collected for biochemical analysis, while two specimens per wound collected for autoradiography. Following collection, the specimens were stored in cold Eagle's Modified Essential Medium (EMEM) media supplemented with 10% fetal calf serum. The biopsy specimens were analyzed as follows.

20 Histologic Evaluation

Histologic specimens were prepared using standard paraffin impregnating and embedding techniques. Four micron sections were made and stained using filtered Harris hemotoxylin and alcoholic eosin; they were then observed under a microscope. All specimens were scored blindly by two investigators at equally distributed points throughout the sections. The widths of the epithelial and connective tissue layers were scored using a grid placed within the ocular of the microscope; the measurement was then converted into millimeters using a micrometer viewed under the same conditions.

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DNA and Protein Determination

DNA determination was performed using a modification of the method of Labarca et al. (1980) Anal. Biochem. 120:344-52. A 50 µl aliquot of tissue extract in concentrated ammonium hydroxide was added to 400 µl of a buffer solution containing 1 M sodium phosphate and 2M sodium chloride (pH 7.0); the pH of the resulting solution was adjusted to 7.4 using HCl. Afterwards, the final solution volume was brought to 500 µl, while maintaining the pH at 7.4. The solution was then added to 2.5 ml of a buffered solution (0.05 M sodium phosphate, 2M sodium chloride, pH = 7.4) of Hoesht dye (1.14 mg/ml). Fluorescence was induced at an excitation wavelength of 352 nm and emission measured at 454 nm. Calf thymus DNA prepared by identical treatment was used to develop standard curves.

Protein content of the tissue extract in concentrated ammonium hydroxide was measured by the Bradford method (Bradford (1976) Anal. Biochem. 72:248-54), with bovine serum albumin as a standard.

Results

The results from histologic evaluation indicated that wounds treated with the combination of purified human PDGF or recombinant PDGF and chemically synthesized human or rat TGF-α had thicker connective tissue and epithelial layers, and more extensive epithelial projections connecting these layers, than wounds receiving no treatment, human or rat TGF-α alone, or pure PDGF alone.

The PDGF/TGF-α treated wounds had greater DNA, protein and collagen contents.

Dosage

To determine the appropriate dosage of purified PDGF, the above-described experiments were repeated

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except that the wounds were treated with 2.5 ng, 5.0 ng, and 10 ng PDGF equivalents of purified PDGF per square millimeter of wound dispersed in 30 μ l of biocompatible gel. The results showed that optimum effects were
5 produced when the PDGF content was 5.0 ng/mm² or higher.

To determine the appropriate dosage of pure PDGF plus TGF- α , combinations in which the weight to weight ratio of PDGF to TGF- α ranged from 1:10 to 25:1
10 were evaluated as described above. Optimum results were achieved with a ratio of between 1:1 and 2:1.

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Claims

1. A method for healing an external wound of a mammal comprising applying to said wound a wound-healing amount of a composition comprising purified platelet-derived growth factor and purified transforming growth factor alpha.
2. The method of claim 1 wherein the weight to weight ratio of said platelet-derived growth factor to said transforming growth factor alpha in said composition is between 1:4 and 25:1.
3. The method of claim 2 wherein said ratio is between 1:2 and 10:1.
4. The method of claim 3 wherein said ratio is about 1:1 or 2:1.
5. A wound healing composition comprising purified platelet-derived growth factor and purified transforming growth factor alpha, in a weight to weight ratio of 1:4 to 25:1.
6. The composition of claim 5 wherein said ratio is between 1:2 and 10:1.
7. The composition of claim 6 wherein said ratio is about 1:1 or 2:1.
8. A method for preparing a composition for healing wounds, comprising mixing purified platelet-derived growth factor and purified transforming growth factor alpha in a weight to weight ratio of between 1:4 and 25:1.

INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US88/04557**

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): **A61K 37/36**

U.S. CL.: **514/2, 3, 21; 530/399**

II. FIELDS SEARCHED

Minimum Documentation Searched *

Classification System	Classification Symbols
U.S.	514/2, 3, 21; 530/399

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *

Chemical Abstracts, "Growth Factors", "Growth Hormones"

III. DOCUMENTS CONSIDERED TO BE RELEVANT *		Relevant to Claim No. 12
Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	
Y	J. TRAUMA, <u>24</u> , No. 9, 549-552 (1984), Grotendorst	1-8
Y	J. Clin. Invest. <u>76</u> , 2323-2329 (1985) Grotendorst	1-8
A	US, A, 4,742,003 (DERYNCK) 03 May 1988	1-8
X	US, H, Serial Number 468,590 (SPORN) 10 June 1983	1-8

* Special categories of cited documents: 10

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IV. CERTIFICATION

Date of the Actual Completion of the International Search

31 March 1989

International Searching Authority

ISA/US

Date of Mailing of this International Search Report

27 APR 1989

Signature of Authorized Officer

Howard E. Schain
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